

Prominin-1/CD133: Lipid Raft Association, Detergent Resistance, and Immunodetection

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SUMMARY

The cell surface antigen prominin-1 (alias CD133) has gained enormous interest in the past 2 decades and given rise to debates as to its utility as a biological stem and cancer stem cell marker. Important and yet often overlooked knowledge that is pertinent to its physiological function has been generated in other systems given its more general expression beyond primitive cells. This article briefly discusses the importance of particular biochemical features of CD133 with relation to its association with membrane microdomains (lipid rafts) and proper immunodetection. It also draws attention toward the adequate use of detergents and caveats that may apply to the interpretation of the results generated. *STEM CELLS TRANSLATIONAL MEDICINE* 2018;7:155–160

SIGNIFICANCE STATEMENT

Prominin-1/CD133, the biological function of which remains to be determined exactly, has aroused a great interest as a stem cell and cancer stem cell marker. Some distinctive features like its differential solubility in nonionic detergents and its association with extracellular vesicles have been unveiled in other cellular systems as CD133 is expressed beyond the stem cell compartment. Given the importance of these phenomena for several aspects of stem cell and cancer stem cell biology, this article clarifies some possible misconceptions and presents often overlooked data that should be useful to the design of future studies in the fields of translational medicine and oncology.

INTRODUCTION

It is now the 20th anniversary of the first description of the murine and human stem cell antigen prominin-1 [1–3]. Since then, this pentaspan membrane glycoprotein, also known as CD133, has been associated to more than 5,000 publications either as the subject or as a biological tool. Indeed, it rapidly aroused great interest as a cell surface biomarker allowing identification and isolation of stem cells from different tissues or organs and as one of the most prominent and most commonly reported cancer stem cell markers [4, 5]. Moreover, CD133 has enabled the unveiling of new features in the biogenesis and maintenance of photoreceptor outer segments since mutations in the *PROM1* gene lead to blindness [6, 7]. Controversies arose however as to its significance as a selective marker of stem cells and with reference to cancer prognosis and progression, partly in relation with its immunodetection using specific epitopes (e.g., AC133 epitope) [8] and for overlooking in this field key data on its general expression beyond stem cells [1, 9–12]. Similarly, its specific biological features like embedding into specific cholesterol-based membrane microdomains (referred to as

lipid rafts) might have promoted misconceptions. The uncovering of molecular and cellular mechanisms regulating the properties of the stem cells might depend on the proper handling of CD133.

Lipid rafts are considered subdomains at nano/micro scales of plasma membranes where the outer membrane leaflet is enriched in cholesterol and sphingolipids. Lipid-lipid and lipid-protein interactions play a major role in their organization [13]. Lipid rafts may serve as specific platforms for signal transduction, and hence participate in a wide range of cellular events under healthy and pathological conditions [14]. They are also involved in membrane trafficking including membrane budding and cell polarization. Membrane-associated proteins involved in these biological events show selective affinity for these membrane subdomains. Furthermore, lipid raft-associated proteins may interact with components of submembraneous cytoskeleton and/or extracellular matrix [15]. The partitioning of certain proteins in and out of lipid rafts could underlie certain human diseases including cancers [14, 15].

Biochemically, lipid rafts appear as distinct liquid-ordered regions of the membrane resistant to extraction in the cold with nonionic detergents, notably Triton X-100 or zwitterionic

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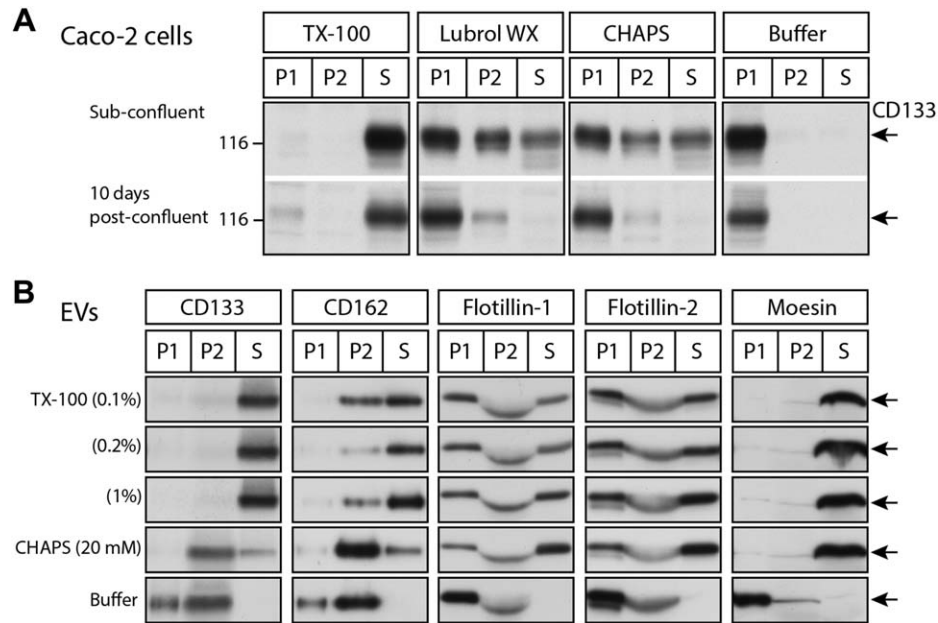


Figure 1. CD133 is soluble in nonionic detergent Triton X-100 but insoluble in Lubrol WX and zwitterionic detergent CHAPS. **(A):** Human Caco-2 cells grown at a subconfluent state or 10 days postconfluence were lysed for 30 minutes on ice in ice-cold solubilization buffer (150 mM NaCl, 2 mM EGTA, 50 mM TRIS-HCl, pH 7.5 plus protease inhibitors) containing either 0.5% (wt/vol) Triton X-100 (TX-100, Sigma-Aldrich), 0.5% Lubrol WX (Lubrol 17A17, Serva) or 20 mM CHAPS (AppliChem GmbH, Darmstadt, Germany) or without detergent (Buffer). Lysates were centrifuged at 4°C first at 16,000g (P1 pellet) for 10 minutes and then the resulting supernatant at 100,000g (P2 pellet) for 1 hour. Proteins in the 100,000g supernatant (S) were concentrated by methanol/chloroform (4:1) precipitation. All fractions were analyzed by immunoblotting for CD133 after SDS-PAGE under reducing condition using mouse monoclonal antibody (mAb) clone 80B258 [12], followed by the appropriate horseradish peroxidase-conjugated secondary Ab (Jackson Immunoresearch) and visualized by enhanced chemiluminescence reagents (ECL system, GE Healthcare Life Sciences). **(B):** EVs released from paramagnetic immuno-isolated CD34⁺ hematopoietic stem and progenitor cells were enriched by ultracentrifugation (200,000g) as described [21] prior to their lysis in solubilization buffer containing Triton X-100 at different concentrations or CHAPS as indicated. Lysates were subjected to differential centrifugation as above. Each fraction was analyzed by immunoblotting for a given protein as specified using the following antibodies: mouse mAb clone KPL-1 (anti-CD162; BD Biosciences), mouse mAb clone 18 and 29 (anti-Flotillin-1 and -2, respectively; BD Biosciences) and rabbit mAb clone EP1863Y (anti-moesin; Abcam). Molecular mass marker (kDa) is indicated. Arrows indicate proteins of interest (Note that hematopoietic stem and progenitor cells were collected from healthy donors after informed consent and approval of the local ethics committee [Ethikkommission an der Technischen Universität Dresden; Ethic board no. EK201092004]). Abbreviations: EV, extracellular membrane vesicle; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

detergent CHAPS [16]. Recently, Gupta and Banerjee delineated a protocol to isolate CD133⁺ lipid rafts from cancer stem cells using the nonionic detergent Triton X-100 [17], but unfortunately without providing supporting data or adequate references. In light of our experience with this glycoprotein and its related molecules, we would like to caution against the use of Triton X-100 for the isolation of CD133⁺ detergent-resistant membrane fractions and for the permeabilization step of CD133 immune-detection in cells or tissues. In view of the widespread utility of CD133 as a cell surface biomarker and the growing interest in lipid rafts in the stem cell and cancer fields, it is important to ascertain appropriate handling in order to generate meaningful data. Therefore, we propose to first remind some distinctive features of CD133 including its biochemical characteristics and then reexamine the detergent resistance of CD133⁺ lipid rafts to different solubilization conditions.

CD133, MEMBRANE PROTRUSIONS, EXTRACELLULAR VESICLES, AND LIPID RAFT ASSOCIATION

A prominent characteristic of CD133 is its restricted association with plasma membrane protrusions irrespective of the nature of cells. In epithelial cells, CD133 is located at the membrane of

microvilli and the primary cilium [1, 18] while in nonepithelial cells CD133 is restricted to finger-like projections that are distributed over the entire surface of human CD34⁺ hematopoietic stem and progenitor cells [11, 19]. Besides membrane protrusions, CD133 is found in endocytic compartments of certain cells such as primitive blood cells and cancer cells [20–23]. Cytoplasmic localization of CD133 in cancer cells might correlate with poor prognosis [24]. Therein, CD133 is associated with small membrane vesicles within multivesicular bodies. The physiological factors regulating the shuttling of CD133 between the plasma membrane and intracellular compartments are poorly described, but the ubiquitination of CD133 and/or its interaction with histone deacetylase 6 or syntenin-1 may be involved [25, 26]. It is worth mentioning that CD133 is also associated with extracellular membrane vesicles (EVs) found in various body fluids under healthy or pathological conditions [27, 28]. The CD133⁺ EVs are either budding from microvilli and/or primary cilia as documented in epithelial cells [18, 29] or released upon the fusion of multivesicular bodies with the plasma membrane, as shown in CD34⁺ primitive blood cells [21]. Irrespective of the molecular mechanisms involved, the release of membrane vesicles needs to be considered when the cellular expression of CD133 is assessed.

The specific retention of CD133 in plasma membrane protrusions of polarized epithelial cells such as Madin-Darby Canine

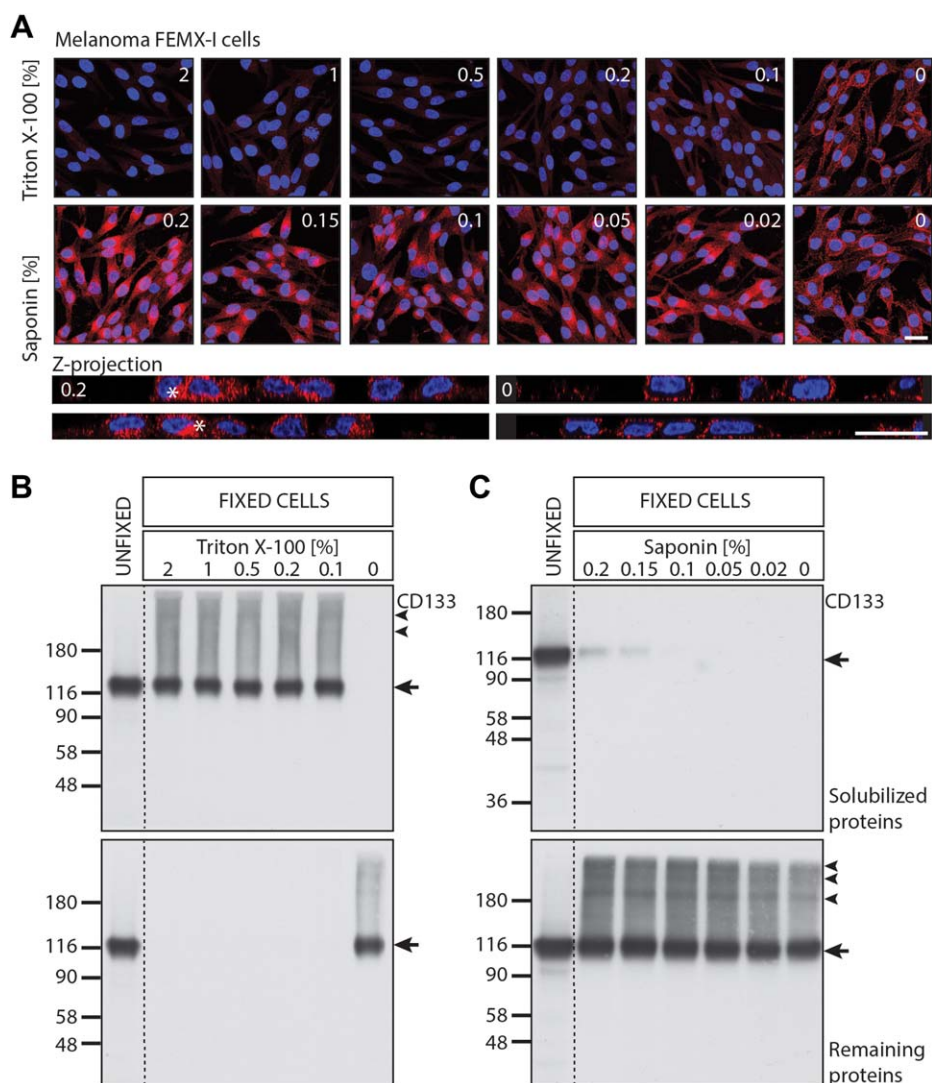


Figure 2. CD133 is extracted by nonionic detergent Triton X-100 from paraformaldehyde (PFA)-fixed melanoma FEMX-I cells. **(A):** FEMX-I cells growing on fibronectin-coated glass coverslips were gently washed with PBS, fixed with 4% PFA for 30 minutes at room temperature and quenched with 50 mM NH₄Cl for 10 minutes prior to permeabilization in PBS containing 0.2% gelatin and various concentrations of Triton X-100 or saponin (AppliChem GmbH) as indicated for 30 minutes. Cells were labeled by indirect immunofluorescence using anti-CD133/1 antibody (AC133 mAb; Miltenyi Biotec) for 45 minutes followed by Alexa-555-conjugated secondary antibody (ThermoFischer Scientific). Nuclei were labeled with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). Samples were mounted in Mowiol 4–88 containing the anti-fading agent 1,4-diazobicyclo-[2.2.2]-octane (Merck-Millipore). All images were acquired using Leica SP5 upright confocal laser scanning microscope, using identical microscope parameters. Composites of 19–24 optical x-y sections (top panels) or z-projections are displayed. Asterisk, intracellular CD133 staining. Scale bar = 25 μm. **(B, C):** FEMX-I cells growing on six-well plates (35 mm) were PFA-fixed prior to permeabilization with 1.5 ml of buffer containing various concentration of Triton X-100 (B) or saponin (C) for 25 minutes at room temperature, as above. Permeabilization buffer was collected from fixed cells and solubilized proteins were concentrated by methanol/chloroform precipitation. In parallel, the fixed cells were scraped, solubilized in RIPA buffer containing 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate and cell lysates prepared. As control, unfixated cells were extracted. The entire materials recovered in the permeabilization buffer (top panels) and half from cell lysates (bottom panels) were separated by SDS-PAGE under nonreducing condition, and analyzed by immunoblotting for CD133 using mAb 80B258. Molecular mass markers (kDa) are indicated. Arrow indicates monomeric CD133 while arrowheads potential PFA-crosslinked oligomers. Abbreviations: PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Kidney cells and the budding of CD133⁺ EVs from human Caco-2 colon-carcinoma-derived cells both require a proper concentration of membrane cholesterol, which directly interacts with CD133 [29–31]. Depletion of cholesterol by methyl-β-cyclodextrin results in redistribution of CD133 over nonprotruding areas of the plasma membrane and/or stimulation of the release of CD133⁺ EVs [29, 31]. These effects are biochemically correlated with the association of CD133 with detergent-resistant membranes.

Microvilli- and EV-associated CD133 were found to be resistant in a cholesterol-dependent manner to solubilization in the mild nonionic detergent Lubrol WX, but not in Triton X-100 [29, 31]. This observation is in deep contrast with the protocol described by Gupta and Banerjee [17]. Insolubility is not related to interaction with the cytoskeleton [31], but may reflect the differential solubilization of components in the inner leaflet of plasma membranes [32]. Subtypes of cholesterol-based membrane

microdomain that are preserved upon Lubrol WX, but not Triton X-100 extraction, are not restricted to epithelial cells but also found in nonepithelial cells [33, 34]. Moreover, certain membrane proteins are specifically incorporated into membrane microdomains that are preserved in Triton X-100 but not Lubrol WX, highlighting the complex organization of the plasma membrane [33] (reviewed in [35]). A recent comparative lipidomic and proteomic study of lipid rafts isolated from human platelets using Triton X-100 versus Lubrol WX has described different profiles of cellular components within the respective fraction of detergent-resistant membranes, further suggesting that the compatibility of the detergent used with the proteins of interest is to be assessed [36].

CD133 DOES NOT ASSOCIATE WITH TRITON X-100 INSOLUBLE MEMBRANE FRACTIONS

To further validate our earlier data, we have extended our observations with human CD133 expressed by cancer Caco-2 cells where it is selectively found in microvilli [10] and CD133⁺ EVs derived from human CD34⁺ hematopoietic stem and progenitor cells [21]. Upon cell lysis at low temperature with Triton X-100 (at concentration of 0.5%) and differential fractionation into supernatant and pellets, almost all of the Caco-2 cell-associated CD133 was recovered as expected in the soluble fraction. In contrast, detergent-resistant membranes containing CD133 were recovered with Lubrol WX (0.5%) or CHAPS (20 mM) (Fig. 1A). Interestingly, the partition to detergent-resistant membranes appeared whether cells were grown at a subconfluent state or 10 days post-confluence (i.e., further differentiated). Yet, higher solubilization with both detergents was evidenced at the subconfluent state suggesting membrane reorganization during enterocytic differentiation, that starts in Caco-2 cells 6–7 days after reaching confluence (Fig. 1A). In CD34⁺ cell-derived EVs, CD133 showed the same susceptibility to Triton X-100 extraction already at 0.1% (Fig. 1B), while CD162 was partly associated with the Triton X-100-resistant membrane, as previously reported [37]. Flotillins marked also resistance to Triton X-100 solubilization while moesin, an adapter protein linking the plasma membrane to the cytoskeleton, was associated with the soluble fraction irrespective of the detergent (Fig. 1B). To sum up, human CD133 is mostly, if not completely, extracted from cells and EVs upon solubilization with Triton X-100. It is also important to keep in mind that different detergents may generate CD133⁺ detergent-resistant membranes originating from cellular membranes other than the plasma membrane (e.g., Golgi membranes, endosomes) in variable proportions [31]. Protocols including isolation of plasma membrane prior to the solubilization step and alternative detergent-free isolation of membrane microdomains have been described [38, 39]. The present data confirm that if one intends to isolate membrane complexes in relation with CD133, the use of Triton X-100 is unlikely to be the detergent of choice. Whenever CD133 is found partly associated with Triton X-100-resistant membrane complexes it is important to assess the significance of this remaining signal, for instance if a link with the cytoskeleton might justify its association with detergent-insoluble complexes.

CD133, SOLUBILITY IN TRITON X-100, AND IMMUNODETECTION

Given that Triton X-100 is frequently used in immunocyto- and histochemistry to permeabilize paraformaldehyde (PFA)-fixed cells,

the high sensitivity of CD133 to solubilization with this detergent prompted us to assess whether this step would interfere with CD133 immunodetection as described for other antigens [40]. The immunodetection of CD133 on fixed melanoma FEMX-I cells [23] was readily impacted upon permeabilization with Triton X-100 at the lowest concentration (0.1%), while barely affected by saponin (Fig. 2A, top panels). Saponin binds to cholesterol within the cell membrane bilayer, leading to saponin-cholesterol complexes and transient pores that are large enough to allow the passage of antibodies through the plasma membrane, as evidenced by the visualization of an intracellular pool of CD133 (Fig. 2A, bottom panels, asterisk). Similar data, that is, the selective loss of CD133 immunoreactivity upon Triton X-100 permeabilization were obtained with Caco-2 cells (data not shown) indicating that this biochemical phenomenon is not restricted to cells growing as individual units but also occurs in polarized monolayers. The reduction, or even the total removal of CD133 immunoreactivity was confirmed by immunoblotting of the proteins extracted by Triton X-100 from PFA-fixed cells. Already at the lowest concentration of Triton X-100 CD133 was totally solubilized (Fig. 2B, top panel), leaving no detectable level of protein in the corresponding cell extracts (Fig. 2B, bottom panel). On the other hand, a permeabilization buffer containing saponin (at a concentration of 0.2% or 0.15%) solubilized a minute fraction of CD133 from the fixed cells (Fig. 2C, top and bottom panels, respectively). We would like to invite researchers to take these observations into account when analyzing the presence of CD133 alone or its association with other antigenic markers using detergents as permeabilizing agents of PFA-fixed cells.

CONCLUSION

The association of CD133 with specific lipid rafts might be of significance in stem cell biology given their implication in signal transduction. This might lead to the concept of “*stem cell-specific lipid rafts*” that would hold determinants necessary for maintaining stem cell properties. Their loss might then promote cell differentiation. Several shedding processes of CD133 that occur during differentiation of neural and hematopoietic progenitor cells or cancer cells like asymmetric cell division [41–43] or the release of EVs might be related to that [10, 21, 27]. The release of CD133⁺ EVs could also mediate communication with the microenvironment, known as stem cell niche [21]. As demonstrated recently, specific exchanges of CD133⁺ lipid rafts mediated by tunneling nanotubes between hematopoietic progenitor cells and leukemic cells may not only promote intercellular communication but also influence the stem cell properties of donor and acceptor cells [44].

It will be of interest to determine the proteome and lipidome of CD133⁺ Lubrol WX-resistant membrane complexes associated with stem cells, differentiated cells or cancerous cells as well as of those associated with EVs derived therefrom. Variations in their composition might be instructive about the cellular state and/or an aberrant transformation. Moreover, EVs as sources of biomaterials might allow noninvasive clinical analyses provided that their lipid raft composition reflects that of the cell of interest under physiological and pathological conditions.

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AUTHOR CONTRIBUTIONS

J.K.: conception and design, performed the experiments, data analysis and interpretation, manuscript writing, final approval of manuscript; A.L. and M.B.: provision of study material or patients, manuscript writing, final approval of manuscript; D.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; C.A.F.: conception and design, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

NOTE ADDED IN PROOF

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